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Expression of Plasmid R388-Encoded Type II Dihydrofolate Reductase as a Dominant Selective Marker in *Saccharomyces cerevisiae*

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The R388 plasmid-encoded drug-resistant type II dihydrofolate reductase gene (R · *dhfr*) was expressed in *Saccharomyces cerevisiae* by fusing the R · *dhfr* coding sequence to the yeast *TRP5* promoter. Yeast cells harboring these recombinant plasmids grew in media with 10 μg of methotrexate per ml and 5 mg of sulfanilamide per ml, a condition which inhibits the growth of wild-type cells. Addition of a 390-base-pair fragment from the 3′-noncoding region of *TRP5* downstream from R · *dhfr* increased expression. Presumably, the added segment promoted termination or polyadenylation or both of the R · *dhfr* transcript. The activity of the plasmid-encoded dihydrofolate reductase and the copy number of the R · *dhfr* plasmid in cells grown in drug-selective media were higher by one order of magnitude than those grown in nutrition-selective media. Plasmid copy number, as well as the plasmid-encoded enzyme level, decreased when cells were selected for prototrophy. In drug-selective media, the plasmid-encoded enzyme level and the content of R · *dhfr* transcripts were nearly constant in cells harboring R · *dhfr* plasmids containing different yeast promoters. In contrast, the plasmid copy number and β-lactamase activity encoded in cis by plasmids were much higher when R · *dhfr* was associated with the weak *TRP5* promoter than when it was fused to the strong *ADC1* promoter. These results indicate that plasmid copy number, i.e., gene dosage of R · *dhfr*, correlates inversely with the strength of the promoter associated with R · *dhfr*, and cells with a higher plasmid copy number were enriched in drug-selective media. The transformation efficiency of R · *dhfr* fused to the *ADC1* promoter was almost the same on drug-selective plates as on nutrition-selective plates, indicating that R · *dhfr* is suitable as a dominant selective transformation marker in *S. cerevisiae*.

Molecular cloning of genes in *Escherichia coli* and other procaryotic organisms has been aided by the development of dominant selective markers. Bacterial clones carrying drug-resistant markers either on plasmid vectors or in the host chromosome can be identified by positive selection on plates containing drugs. Drug-resistant markers have also been useful when used to isolate plasmid copy number mutants (30) or promoter mutants (3) in *E. coli*. The development of plasmid vectors capable of replicating autonomously in *Saccharomyces cerevisiae* (5, 18, 26, 28) and the existence of procedures for transformation of yeast cells (15, 16) have greatly facilitated molecular cloning in *S. cerevisiae*. To identify yeast cells transformed by plasmid vectors, we developed a dominant selection system with R388 plasmid-encoded type II dihydrofolate reductase (R · *dhfr*).

Generally, in eucaryotic cells, dTMP is synthesized from dUMP (de novo pathway) or thymidine (salvage pathway) (Fig. 1). Since *S. cerevisiae* lacks thymidine kinase (14), dTMP is synthesized only by the de novo pathway in which methylenetetrahydrofolate, which is required for the conversion of dUMP to dTMP, is synthesized from dihydrofolate via tetrahydrofolate. Dihydrofolate reductase (DHFR), the enzyme that catalyzes the conversion of dihydrofolate to tetrahydrofolate, is inhibited by aminopterin or methotrexate (9, 19, 32). *S. cerevisiae* is susceptible to these drugs when the de novo synthesis of dihydrofolate is blocked by sulfanilamide (9, 19, 32), a compound which inhibits conversion of hydroxymethyldihydropterin pyrophosphate to dihydropterin (Fig. 1). Naturally occurring plasmid R388-encoded type II DHFR in *E. coli* is insensitive to trimethoprim, aminopterin, or methotrexate (2). The enzyme is a tetramer with a monomer molecular weight of ca. 8,500 (23, 29). Restoration of yeast growth in the presence of methotrexate and sulfanilamide should occur if R · *dhfr* is expressed in *S. cerevisiae*. This was achieved by fusing the R · *dhfr* coding sequence with the yeast *TRP5* or *ADC1* promoters and terminators. The effects of dominant selection on plasmid copy number and on the expression of the gene product encoded by the plasmid in cis are also described.

MATERIALS AND METHODS

Bacterial and yeast strains. *E. coli* MC1061 [*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsr rpsL*] was used as a host strain for plasmid preparation. *S. cerevisiae* SHY3 (**a** *steCV9 ura3-52 trp1-289 leu2-3 leu2-112 his3-Δ1 ade1-100 can1-100*) (7) was used throughout this work.

Plasmids. *S. cerevisiae-E. coli* shuttle vectors YRp7 (28) and YCp19 (25) were obtained from R. Davis. A unique *Pvu*II site in YRp7 was converted to *Kpn*I and used as YRp7-K in this work. YEp13 (10) was obtained from M. Casadaban. pYe(*trp1 trp5*) (33) and pYe(*CEN3*)30 (13) were obtained from J. Carbon. pAAH5 (1) was obtained from B. Hall. pSV2*dhfr*, constructed by attaching *Hin*dIII linkers to the *Taq*I site 42 base pairs (bp) upstream from the R · *dhfr* ATG codon (29), was obtained from B. Howard.

Enzymes, chemicals, and media. Restriction endonucleases, DNA polymerase I Klenow fragment, and bacteriophage T4 DNA ligase were purchased from Bethesda Research Laboratories. Radioactive nucleotides were from Amersham Corp. Dihydrofolate, methotrexate, sulfanilamide, and NADPH were obtained from Sigma Chemical Co. Nitrocefin was from Glaxo Research. Rich medium (YPD) contained 2% Bacto-Peptone, 1% yeast extract, and

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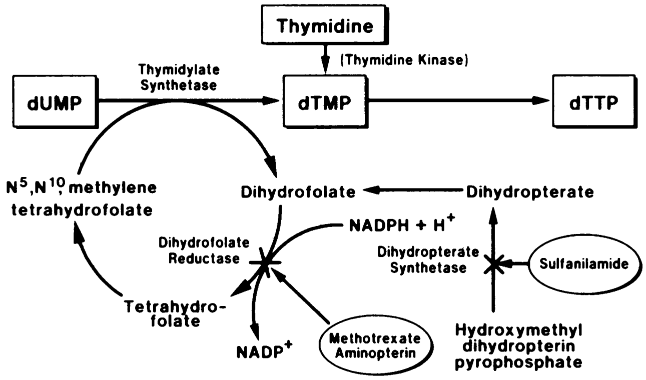

FIG. 1. Pathways for the synthesis of dTMP in *S. cerevisiae*.

2% glucose. YPD-2 medium contained 0.25% Bacto-Peptone, 1% yeast extract, and 2% glucose. Synthetic medium (SD) contained 0.67% yeast nitrogen base without amino acid (Difco Laboratories) and 2% glucose. For the SHY3 strain, 50 μg each of histidine, leucine, tryptophan, adenine, and uracil per ml was added. For the growth of SHY3 cells carrying YRp7 derivatives, SD medium was supplemented with 0.5% Casamino Acids and 50 μg each of adenine and uracil per ml. For the growth of SHY3 cells carrying YEp13 derivatives, SD medium was supplemented with 50 μg each of tryptophan, histidine, adenine, and uracil per ml.

Yeast transformation. Yeast transformation was performed by either a spheroplast (15) or lithium acetate method (16). Drug-resistant transformants of SHY3 strains were directly selected by the lithium acetate method on SD medium containing 0.5% Casamino Acids; 50 μg each of uracil, adenine, and tryptophan per ml; 5 mg of sulfanilamide per ml; and 10 μg of methotrexate per ml. Transformation efficiency of SHY3 strains grown in nutrition-selective media was ca. 1,000 colonies per μg of R · *dhfr* plasmid DNA.

Enzyme activities. Exponentially growing cells were harvested when the optical density at 600 nm (OD_{600}) was equal to 1, and then they were washed with water and suspended in three volumes of 50 mM potassium phosphate (pH 7.0) containing 1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride, and 1 μg each of leupeptin and pepstatin per ml. Cells disintegrated by shaking with glass beads were centrifuged at 12,000 rpm for 30 min in a Sorval SS34 rotor, and the supernatant was assayed for β-lactamase. For the DHFR assay, cell extract fractionated by ammonium sulfate (40 to 80% saturation) was used as an enzyme source.

β-Lactamase was assayed colorimetrically with nitrocefin (21). The reaction mixture (0.5 ml), containing 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM β-mercaptoethanol, 20 μg of nitrocefin, and 100 to 900 μg of yeast cell extract, was incubated at 23°C. The decrease in absorbance at 386 nm was monitored. One enzyme unit was defined as the decrease of 1 OD_{386} unit per 10 min at 23°C.

DHFR activity was measured by the reduction of dihydrofolic acid (DHFA) to tetrahydrofolic acid. The reaction mixture (0.4 ml), containing 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 15 mM β-mercaptoethanol, 75 μM NADPH, 50 μM DHFA, 25 μM methotrexate, and ca. 200 μg of extract, was incubated at 23°C. The absorbance at 340 nm was monitored. The DHFA-independent NADPH oxidase activity was subtracted, and one enzyme unit was defined as 1 nmol of DHFA reduced per min at 23°C.

Preparation of RNA. RNA was prepared by the guanidine thiocyanate method (11). Exponentially growing cells washed with water were suspended in a guanidine solution containing 6 M guanidine thiocyanate, 5 mM sodium citrate, 0.5% sodium lauroylsarcosine, 1% β-mercaptoethanol, and 1 drop of Antifoam A (Sigma Chemical Co.) and were disrupted by shaking with glass beads. A clear supernatant obtained by centrifugation at 12,000 rpm for 15 min was loaded onto a 5.7 M CsCl cushion and centrifuged at 28,000 rpm for 20 h to precipitate the RNA. RNA dissolved in 10 mM Tris-hydrochloride (pH 7.6) containing 1 mM EDTA and 0.05% sodium dodecyl sulfate (SDS) was treated with phenol and precipitated with two volumes of ethanol. Polyadenylated [Poly(A)$^+$] RNA was prepared with oligodeoxythymidylate cellulose (4).

Electrophoresis and hybridization. DNA was separated by electrophoresis on a 0.8% agarose gel in 40 mM Tris-acetate (pH 8.1), containing 2 mM EDTA, and transferred to nitrocellulose filters. Glyoxylated RNA was separated by electrophoresis on a 1.5% agarose gel in 10 mM sodium phosphate (pH 7.0) and transferred to nitrocellulose filters. DNA-DNA and DNA-RNA hybridization was performed in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, [pH 7.4], and 1 mM EDTA) containing 50% formamide, 5× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone) and 100 μg of sonicated salmon sperm DNA per ml at 42°C; filters were washed with 2× SSPE (20). SDS-polyacrylamide gel electrophoresis was carried out in a 15% polyacrylamide gel containing 0.1 M sodium phosphate (pH 7.2), 0.1% SDS, and 6 M urea. The protein concentration was determined by the method of Bradford (8), with bovine serum albumin as a standard.

RESULTS

Growth inhibition of *S. cerevisiae* by methotrexate and sulfanilamide. Growth of *S. cerevisiae* strain SHY3 in SD medium supplemented with 0.5% Casamino Acids and 50 μg each of tryptophan, adenine, and uracil per ml was not significantly affected by 10 μg of methotrexate per ml (9). However, growth was almost completely inhibited by 2.5 μg of methotrexate per ml in the presence of 5 mg of sulfanilamide per ml (Fig. 2A). In 10 μg of methotrexate per ml, sulfanilamide at concentrations above 300 μg/ml completely inhibited growth (Fig. 2B). Therefore, for the selection of drug-resistant transformants, methotrexate and sulfanilamide at 10 μg/ml and 5 mg/ml, respectively, were included in the SD medium supplemented with 0.5% Casamino Acids and 50 μg each of tryptophan, adenine, and uracil (standard drug-selective medium). Similarly, in YPD-2 medium, growth of *S. cerevisiae* was not affected by 80 μg of methotrexate per ml, whereas it was completely inhibited by 40 μg of methotrexate per ml in the presence of 5 mg of sulfanilamide per ml (data not shown). Since tetrahydrofolate is required for the synthesis of adenine, glutamic acid, glycine, and methionine, complete removal of these components from the drug medium may cause severe growth inhibition (9, 32).

Construction of R · *dhfr* plasmids. The R · *dhfr* coding sequence flanked by 800- and 100-bp noncoding regions at the 5′ and 3′ sides, respectively, was subcloned into the *S. cerevisiae-E. coli* shuttle vector YEp13. Yeast strain SHY3 was transformed by the R · *dhfr* plasmid and selected by complementation for leucine independence, and expression of R · *dhfr* was examined on plates containing the standard drug-selective medium. Since the transformant harboring the

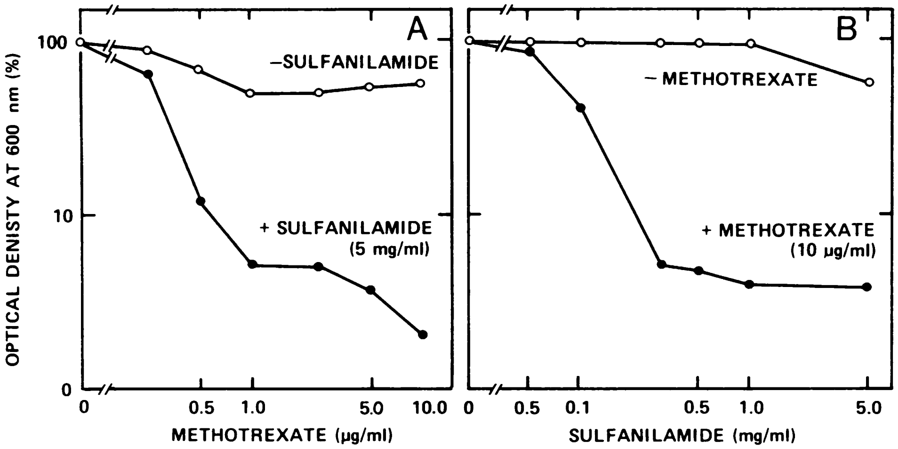

FIG. 2. Effect of methotrexate and sulfanilamide on yeast growth. A fresh saturation culture of SHY3 (5 µl) was added to 2 ml of SD medium containing 0.5% Casamino Acids; 50 µg each of tryptophan, adenine, and uracil per ml; and various concentrations of the drugs. After 4 days of incubation at 30°C in the dark, the OD_{600} was measured. The OD_{600} of the culture without drugs is defined as 100%. (A) Methotrexate in the presence or absence of sulfanilamide. (B) Sulfanilamide in the presence or absence of methotrexate.

R · *dhfr* plasmid was unable to grow (data not shown), a requirement for a yeast expression sequence, such as a promoter or a terminator, was suggested. Therefore, various plasmids carrying the fusion of R · *dhfr* and yeast promoters were constructed and their properties examined (Fig. 2 and Table 1). Transformants selected with a nutritional marker were examined for expression of R · *dhfr* by growth of cells on plates under the standard drug-selective conditions.

Expression of R · *dhfr* fused to the *TRP5* promoter and the restoration of growth in drug-selective media. Fusion of R · *dhfr* with the yeast *TRP5* promoter region was accomplished based on the nucleotide sequence of the coding region and the three transcription starting sites identified in vivo (33). The R · *dhfr* coding region was linked to the *TRP5* promoter region at a unique *Cla*I site downstream from the first starting site (33). The plasmid pTD1 (Fig. 3) carries the yeast *TRP5* promoter and the first transcription-starting site fused to the R · *dhfr* coding sequence 42 bp upstream from the ATG codon. This plasmid also has *TRP1* and *URA3* as selective markers and *ARS1* as a yeast replication origin. SHY3 cells, transformed with pTD1 by selecting for tryptophan independence, were examined for drug resistance. Trp^+ transformants could grow in the standard drug-selective medium, but growth was very slow (Table 1).

TABLE 1. Growth properties and transformation efficiencies of various R · *dhfr* plasmids in the drug-selective medium[a]

Plasmid[b]	Origin	*CEN*	Nutritional marker	Promoter	Terminator	Drug resistance		Doubling time (h)		Transformation efficiency (%)[c]	
						A	B	A	B	A	B
pTD1	*ARS1*		*TRP1*, *URA3*	*TRP5*		+	+	9	18	1	1
pTD2	*ARS1*	*CEN4*	*TRP1*	*TRP5*		+	–				
pTD4	*ARS1*	*CEN3*	*TRP1*	*TRP5*		+	–			2	
pTD3	2µ, *ARS1*		*LEU2*	*TRP5*		+	+				5
pTDT1	*ARS1*		*TRP1*, *URA3*	*TRP5*	*TRP5*	+	+	6.5	12	17	6
pADA1	2µ		*LEU2*	*ADC1*	*ADC1*	+	+				48
pADA3	*ARS1*		*TRP1*	*ADC1*	*ADC1*	+	+	5	10	94	50
pADA4	*ARS1*	*CEN3*	*TRP1*	*ADC1*	*ADC1*	+	+	4	7	80	47
pGDT3	*ARS1*		*TRP1*, *URA3*	*GAL1*	*TRP5*	+	+				
PADA2	Chromosome integration			*ADC1*	*ADC1*	+	+	4	7		

[a] Cells were grown in SD medium containing 0.5% Casamino Acids; 50 µg each of tryptophan, adenine, and uracil per ml; 10 µg of methotrexate per ml; and 0.5 mg of sulfanilamide (A) or 5 mg of sulfanilamide (B) per ml, except for cells with pGDT3 which were grown in synthetic medium containing galactose and the drugs without glucose.

[b] The R · *dhfr* plasmids were constructed as follows.

[c] Transformation efficiency of the SHY3 strain grown in nutrition-selective medium was ca. 1,000 colonies per µg of plasmid DNA; transformation efficiency on drug plates is expressed as a percentage of the auxotrophic selection. pTD2, a *Bam*HI-*Eco*RI fragment (0.9 kb) carrying the *TRP5* promoter-R · *dhfr* coding region was isolated from pTD1 (see Fig. 3) and cloned into the *Pvu*II site of YCp19 (25); pTD4, a *Bam*HI-*Cla*I fragment (0.9 kb) carrying *CEN3* was isolated from pYe(CEN3)30 (13) and inserted into the *Bam*HI-*Pvu*II sites of pTD1; pTD3, a *Bam*HI-*Hin*dIII fragment (1.5 kb) carrying the *TRP5* promoter R · *dhfr* coding sequence and *ARS1* was isolated from pTD1 and cloned into the *Bam*HI-*Hin*dIII site of YEp13 (10); pADA3, a *Bam*HI fragment (2.4 kb) carrying the *ADC1* promoter-R · *dhfr ADC1* terminator was isolated from pADA1 (see Fig. 3) and inserted into the *Bam*HI site of YRp7-K; pADA4, the same *Bam*HI fragment used for construction of pADA3 was inserted into the *Bam*HI site of pYe(CEN3)30 (13); pGDT3, a 0.8-kb fragment carrying the *GAL1* promoter and transcription initiation site but lacking the ATG codon was isolated from pBM125 (obtained from M. Johnston, Stanford University) and cloned into pTDT1 to replace the *Bam*HI-*Cla*I fragment carrying the *TRP5* promoter; pADA2, the same *Bam*HI fragment used for the construction of pADA3 was inserted into the *Bam*HI site of pUC8 (31).

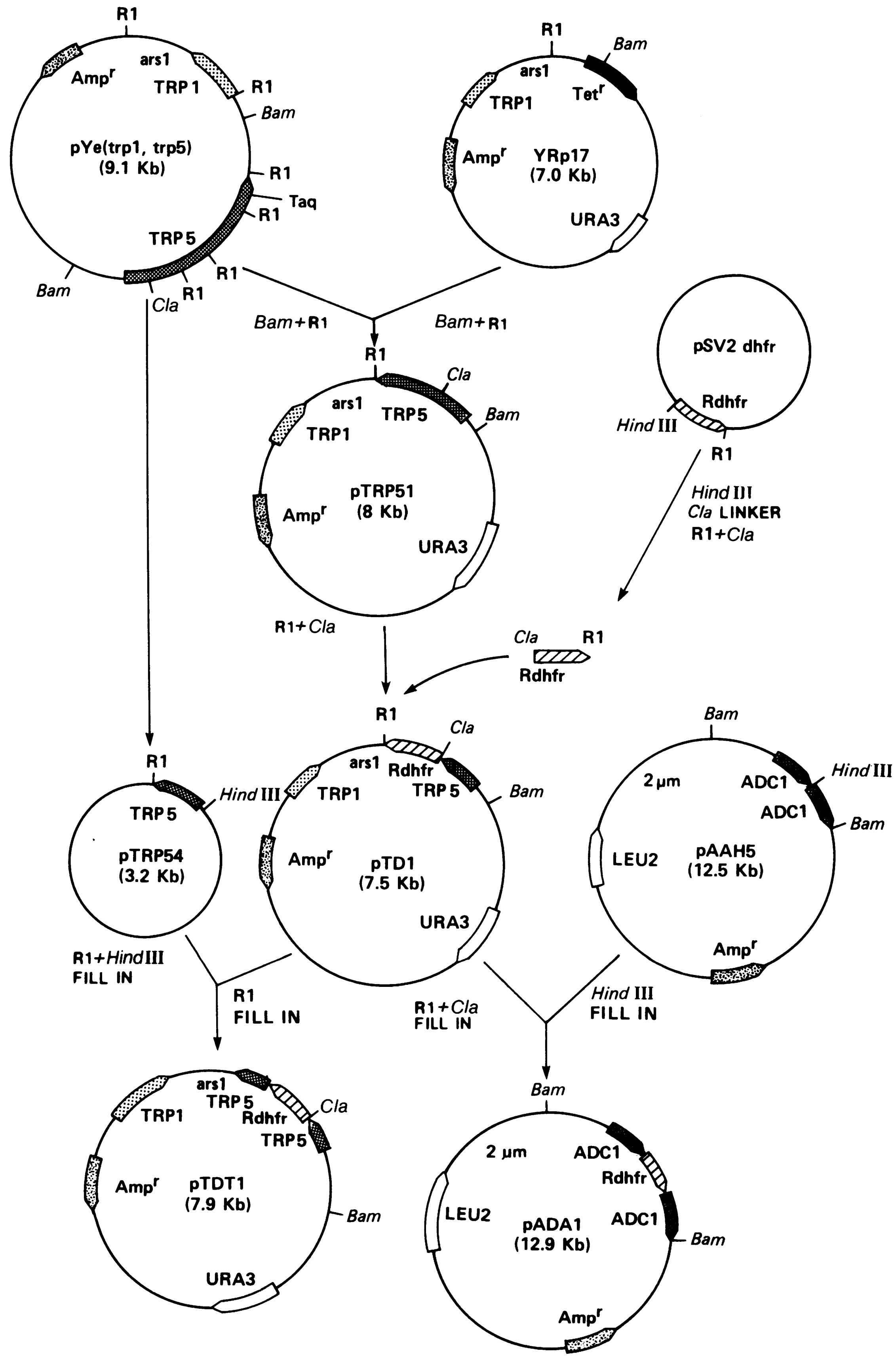

FIG. 3. Construction of R · *dhfr* plasmids associated with the *TRP5* and *ADC1* promoters. pTRP51 was constructed by insertion of a 1.3-kb *Eco*RI-*Bam*HI fragment of pYe (*trp1 trp5*) containing the *TRP5* promoter and a 780-bp coding sequence into *Eco*RI-*Bam*HI-cleaved

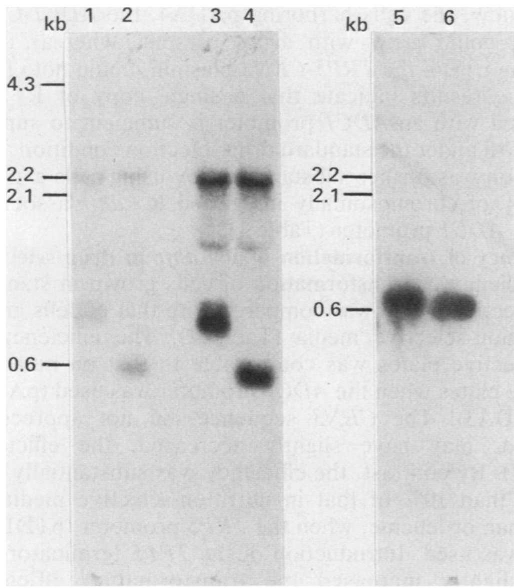

FIG. 4. Northern analysis of total and poly(A)$^+$ RNA for R · *dhfr* transcripts. Total and poly(A)$^+$ RNA were prepared as described in the text. Glyoxylated RNA was separated by 1% (lanes 1 through 4) or 1.5% (lanes 5 and 6) agarose gel electrophoresis. RNA transferred to nitrocellulose filters was hybridized with the nick-translated *Cla*I-*Eco*RI fragment carrying the R · *dhfr* gene (Fig. 3). Lane 1, 24 μg of total RNA of SHY3(pTD1); lane 2, 24 μg of total RNA of SHY3(pTDT1); lane 3, 13 μg of poly(A)$^+$ RNA of SHY3(pTD1); lane 4, 13 μg of poly(A)$^+$ RNA of SHY3(pTDT1); lane 5, 25 μg of total RNA of SHY3(pADA1); and lane 6, 1 μg of poly(A)$^+$ RNA of SHY3(pADA1).

Termination and polyadenylation of the transcript promoted by the 3′-noncoding region of *TRP5*. Total and poly(A)$^+$ RNA fractions prepared from SHY3 cells harboring pTD1 in the absence of drug selection media were fractionated by agarose gel electrophoresis and were transferred to nitrocellulose. By using both total and poly(A)$^+$ RNA, the R · *dhfr* probe hybridized with a transcript of about 1 kilobase (kb) (Fig. 4). Since the R · *dhfr* coding and 3′-noncoding regions are 237 and ca. 100 bp, respectively, the results indicate that the R · *dhfr* transcript terminates within the *TRP1-ARS1* segment. To examine the effect of the 3′ side of the *TRP5* noncoding region on R · *dhfr* expression, a *Taq*I-*Eco*RI fragment derived from the 3′ region and containing 20 bp of the *TRP5* coding region at the COOH-terminus (33) was placed downstream of R · *dhfr* (pTDT1 in Fig. 3). The cells harboring pTDT1 grew faster than those carrying pTD1 (Table 1) and were seen to be more resistant to drugs; a R · *dhfr* transcript about 650 nucleotides in length with a poly(A)$^+$ tail (Fig. 4) was produced. This result indicates that the *TRP5* 3′-noncoding region has signals for transcription termination and polyadenylation. From the size of the R · *dhfr* transcript, the signal for polyadenylate addition might be located about 100 to 200 bp downstream from the *TRP5* termination codon. The transcripts of about 2.2 kb in length, detected both in pTD1 and pTDT1 (Fig. 4), were not characterized further.

TABLE 2. R · DHFR and β-lactamase activities in yeast extract[a]

Plasmid	Activities for the following enzymes with the indicated drug selection:[b]			
	R · DHFR		β-Lactamase	
	−	+	−	+
YRp7-K			0.3	
pTD1	0.2	1.1	0.3	2.0
pTDT1	0.2	2.1	0.3	1.1
pADA3	0.1	1.5	0.1	0.5
No plasmid			<0.1	

[a] SHY3 cells carrying plasmids were grown in SD medium supplemented with 0.5% Casamino Acids and 50 μg each of uracil and adenine per ml, or in SD medium containing 0.5% Casamino Acids, 50 μg each of uracil and adenine per ml, 10 μg of methotrexate per ml, and 5 mg of sulfanilamide per ml. Yeast extract was prepared and assayed as described in the text.

[b] Activities are expressed as units per milligram of protein, and 1 unit of R · DHFR and β-lactamase corresponds to 1 nmol of DHFA reduced per min at 23°C and 1 OD_{386} of nitrocefin reduced per 10 min at 23°C, respectively.

Expression of R · *dhfr* fused with the *ADC1* promoter-terminator. Alcohol dehydrogenase I is among the most abundant proteins in *S. cerevisiae*, and *ADC1* mRNA comprises about 1% of poly(A)$^+$ RNA (6). We compared the strong *ADC1* promoter-terminator system with the relatively weak *TRP5* promoter-terminator system. Both pADA1 and pADA3, each carrying the R · *dhfr* coding region placed between the *ADC1* promoter and terminator, could confer drug resistance to *S. cerevisiae* (Fig. 3 and Table 1). Cells harboring pADA3 grew much faster than those with pTDT1 (Table 1). Cells with pADA1 contained more poly(A)$^+$ R · *dhfr* message than those with pTDT1 grown in nutrition-selective media (Fig. 4). As will be described later, these differences are probably due to the difference in promoter strength between *ADC1* and *TRP5* since the plasmid copy number was nearly the same (Table 2).

Plasmid-encoded DHFR activity and drug selection. The expression of R · *dhfr* in *S. cerevisiae* was directly confirmed by measuring the methotrexate-resistant DHFR activity in extracts prepared from cells harboring R · *dhfr* plasmids grown in the presence of drugs (see above). The specific activities of methotrexate-resistant DHFR in pTD1, pTDT1, and pADA3 extracts were found to be nearly the same (Table 2). Analysis of the extracts by SDS-polyacrylamide gel electrophoresis revealed the presence of the expected 8,500-dalton polypeptide (23) (data not shown). However, methotrexate-resistant enzyme activity was hardly detectable in cells grown in the nutrition-selective medium (Table 2). These results indicate that in drug-selective media,

YRp17. pSV2*dhfr* was cleaved with *Hin*dIII and blunt-ended with DNA polymerase I Klenow fragment. *Cla*I linker was attached to the filled-in *Hin*dIII site and cleaved with *Cla*I and *Eco*RI. A 0.4-kb *Cla*I-*Eco*RI fragment carrying R · *dhfr* was isolated by agarose gel electrophoresis and inserted into the *Cla*I-*Eco*RI site of pTRP51 to yield pTD1. The *Taq*I-*Eco*RI (0.4 kb) fragment of pYe (*trp1 trp5*) carrying the *TRP5* terminator was isolated by agarose gel electrophoresis and subcloned into the *Acc*I-*Eco*RI site of pUC8 (31) to yield pTRP54. pTRP54 was cleaved with *Hin*dIII and *Eco*RI and filled in with the Klenow fragment. pTDT1 was obtained by insertion of the filled-in *Hin*dIII-*Eco*RI fragment (0.4 kb) carrying the *TRP5* terminator into the filled-in *Eco*RI site of pTD1. pADA1 was constructed by insertion of the filled-in *Cla*I-*Eco*RI fragment (0.4 kb) carrying R · *dhfr* (derived from pTD1) into the filled-in *Hin*dIII site of pAAH5.

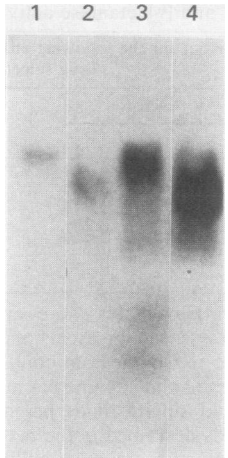

FIG. 5. Northern analysis of R · *dhfr* transcripts. Total RNA was prepared as described in the text. About 20 μg of RNA was separated on agarose gels and hybridized with R · *dhfr* probe as described in the legend to Fig. 4. SHY3(pTD1) (lanes 1 and 3) or SHY3(pADA3) (lanes 2 and 4) were grown in either nutrition-selective medium (lanes 1 and 2) or drug-selective medium (lanes 3 and 4).

the population of yeast cells containing high R · DHFR activity is enriched.

Level of R · *dhfr* transcripts in drug-selective media. The levels of R · *dhfr* transcripts in the drug-selective medium were markedly higher than those in the nutrition-selective medium with all the R · *dhfr* plasmids tested (Fig. 5). These results indicate that yeast cells containing a high level of R · *dhfr* mRNA were enriched in the drug-selective medium and that growth of cells containing drug-resistant enzyme activity below some critical level was inhibited.

Increased plasmid copy number in drug-selective media. The copy number of plasmids in drug- and nutrition-selective media was compared by Southern analysis; in all cases, the apparent copy number was much higher when cells were grown in the presence of drugs (Fig. 6). The elevated copy number decreased reversibly when the cells were grown under nutrition-selective conditions. However, it should be noted that the copy number of pADA3, which was fused with the *ADC1* promoter, was substantially lower than those of pTD1 and pTDT1 associated with the *TRP5* promoter. These observations were confirmed in extracts by another indicator of the plasmid copy number: β-lactamase activity encoded by the plasmids in cis. The specific activity of β-lactamase increased as a function of drug concentration (Fig. 7). The low level of β-lactamase activity under conditions of auxotrophic selection, which was almost the same with different plasmids, increased by one order of magnitude when the cells were grown with drugs; however, the activities of pTD1 and pTDT1 were significantly higher than that of pADA3 (Table 2). These results indicate that the high level of R · *dhfr* expression in drugs was determined by at least two factors which were correlated inversely: the copy number of plasmids, i.e., R · *dhfr* gene dosage, and the strength of the promoter associated with R · *dhfr*.

Effect of the *CEN* sequence on drug selection. The growth of the cells in the presence of drugs should be a measure of the promoter strength if the plasmid copy number is constant. This was tested by introducing into pTD1 and pADA3 the *CEN* sequence, which is known to stabilize *ARS* plasmids and keep the copy number near one per cell (13). In support of this view, the cells harboring pADA4, the *ADC1-CEN3* plasmid, could grow with drugs present, whereas those harboring pTD4, the *TRP5-CEN3* plasmid, could not (Table 1). These results indicate that a single copy of R · *dhfr* associated with an *ADC1* promoter is sufficient to support cell growth under the standard drug selection condition. This conclusion was further substantiated by using cells carrying one copy of chromosomally integrated R · *dhfr* associated with the *ADC1* promoter (Table 1).

Efficiency of transformation of R · *dhfr* in drug selection. The efficiency of transformation of cells grown in standard drug-selective media was compared with that of cells grown in nutrition-selective media (Table 1). The efficiency on drug-selective plates was comparable to that on nutrition-selective plates when the *ADC1* promoter was used (pADA1 and pADA3). The *CEN3* sequence did not appreciably affect, or may have slightly decreased, the efficiency (pADA4). By contrast, the efficiency was substantially lower, less than 10% of that in nutrition-selective media for tryptophan or leucine, when the *TRP5* promoter (pTD1 and pTD3) was used. Introduction of the *TRP5* terminator into pTD1 slightly increased the transformation efficiency (pTDT1). Thus, the apparent transformation efficiency seems to be correlated with the strength of the promoter associated with the R · *dhfr* coding sequence.

DISCUSSION

Several bacterial genes, such as *leuB* (27), Tn*601* (17), and Tn*5* (unpublished data), that encode neomycin resistance and β-lactamase (22) have been expressed in *S. cerevisiae* without further manipulation of the yeast regulatory sequence. By contrast, expression of R · *dhfr* required appropriate

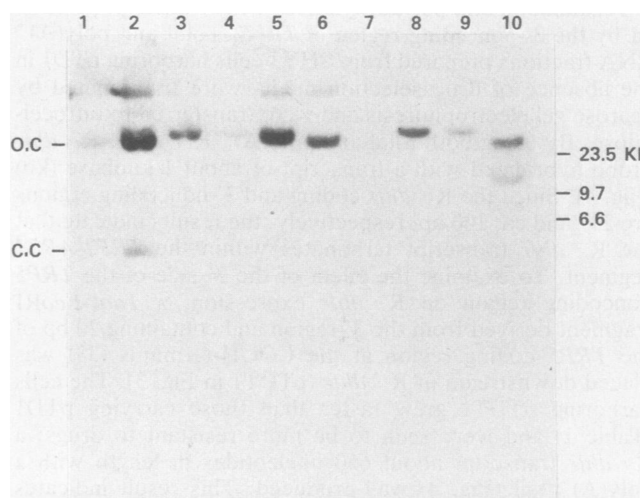

FIG. 6. Southern analysis of R · *dhfr* plasmids. Yeast DNA was prepared as described previously (12) and separated by 0.8% agarose gel electrophoresis. DNA transferred to nitrocellulose filters was hybridized with nick-translated pBR322 DNA. SHY3 cells carrying R · *dhfr* plasmids were first grown to saturation in the synthetic medium lacking tryptophan (lanes 1, 4, and 7), and small portions (0.1%) were inoculated into standard drug medium (lanes 2, 5, and 8). When the cultures reached saturation, 0.1% portions were inoculated into synthetic medium lacking tryptophan and grown to saturation (lanes 3, 6, and 9). Lanes 1 through 3, pTD1; lanes 4 through 6, pTDT1; lanes 7 through 9, pADA3; lane 10, DNA molecular weight standards. O.C, Open circular form; C.C, closed circular form.

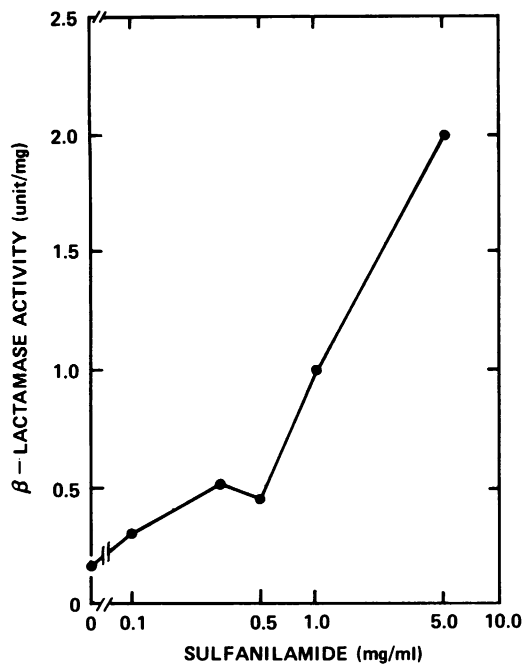

FIG. 7. Effect of sulfanilamide on the specific activity of β-lactamase in SHY3(pTD1). SHY3(pTD1) was grown in SD medium containing 0.5% Casamino Acids; 50 μg each of adenine and uracil per ml; 10 μg of methotrexate per ml; and various concentrations of sulfanilamide. Extracts were prepared and assayed for R · DHFR as described in the text.

fusion of the coding sequence with the *TRP5* promoter. The methotrexate-resistant DHFR activity and the polypeptide product (molecular weight, 8,500) detected in *S. cerevisiae* carrying R · *dhfr* plasmids indicated that R · *dhfr* was expressed in *S. cerevisiae*. The size of R · *dhfr* mRNA directed by pTD1 indicated that transcription was terminated within the *TRP1-ARS1* fragment. Since the direction of transcription of *TRP1* is opposite that of R · *dhfr*, the presence of a terminator-like sequence within the *TRP1*-ARS1 segment is suggested. Presumably, yeast enzyme systems do not recognize the *E. coli* transcription-termination signal downstream of the R · *dhfr* coding region.

The sequence TAG-(37 bp)-TAGT-(11 bp)-TTTT-(6 bp)-TTT which is 80 bp downstream of the *TRP5* termination codon (33) is analogous to the consensus sequence for yeast transcription termination (34). The *TRP5* 3'-flanking region containing this sequence appears to carry sufficient information for termination and polyadenylation, because these processes were promoted when the fragment was placed downstream of R · *dhfr*. Improved growth and the elevated R · DHFR activity of yeast cells harboring pTDT1 suggested that termination and polyadenylation of the R · *dhfr* transcript near the termination codon increased expression.

The copy number of R · *dhfr* plasmids in cells in drug-selective media was much higher than in cells grown in nutrition-selective media. In view of the instability of *ARS1* or 2μ plasmids, it is tempting to speculate that the cells which lost the R · *dhfr* plasmid due to unequal plasmid segregation during cell division (13) were killed. The apparent lack of effect of the drugs on cells carrying integrated R · *dhfr* in the chromosome is consistent with this view. The growth rate of cells harboring various *dhfr* plasmids (Table 1) might be determined primarily by the promoters associated with the R · *dhfr* coding sequence. Only a small population of cells carrying weak promoter plasmids are able to grow in drug-selective media since they require a high plasmid copy number for growth.

The results described above indicate that R · *dhfr* is useful as a positive selective marker in *S. cerevisiae*; some of its potential uses are as follows.

(i) Selective marker in *S. cerevisiae* transformation. Drug-resistant transformants can be isolated directly on plates containing methotrexate and sulfanilamide. The efficiency of transformation depends on the strength of the promoter associated with R · *dhfr* (Table 1) and on the concentration of the drugs employed, i.e., the selective pressure. In keeping with these results, a transformation efficiency of yeast cells nearly comparable to that observed with pADA3 was also achieved on G418 plates when the Tn*5*-derived neomycin resistance gene was associated with the *ADC1* promoter and terminator as a selective marker (unpublished data). We isolated several yeast replicator sequences (*ARS*) from a genomic DNA library established in pADA2 by using R · *dhfr* selection (unpublished data).

(ii) Isolation of regulatory mutants. The growth of cells in the presence of drugs is a measure of R · *dhfr* expression and is useful for studying gene expression. For example, we have fused the *GAL1* promoter (24) with R · *dhfr* which enables cells to grow only on galactose medium in the presence of drugs. After prolonged growth, many drug-resistant colonies were obtained by plating the cells on drug-selective media containing glucose (unpublished data). It may be possible to isolate *GAL1* promoter mutants from these strains.

(iii) Isolation of copy number mutants. Since pTD2 and pTD4, which carry *CEN* sequence, did not confer yeast drug resistance under standard conditions, it may be possible to isolate *CEN* mutants by isolating drug-resistant cells. Such experiments are currently under way in our laboratory.

(iv) Overproduction of gene products cloned into yeast plasmid vectors. Enrichment of cells with higher plasmid copy numbers by drug selection is useful for overproduction of proteins encoded by nonselectable genes cloned into yeast vectors, as evidenced for the gene coding for β-lactamase. We are currently optimizing the expression of cDNA by using selectable markers with weak promoters and cloning cDNA segments under the control of strong promoters.

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